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Authors:

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Analysis of fermentation selectivity of purified galacto-oligosaccharides by *in vitro* human faecal fermentation

Barbara Rodriguez-Colinas[a], Sofia Kolida[b], Magdalena Baran[b], Antonio O. Ballesteros[a] , Robert A. Rastall[b] and Francisco J. Plou[a,*]

[a] *Instituto de Catalisis y Petroleoquimica, CSIC, 28049 Madrid, Spain*

[b] *Department of Food and Nutritional Sciences, University of Reading, PO Box 226, Whiteknights, Reading, RG6 6AP, UK*

* ***Corresponding author****:* Francisco J. Plou, Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Cantoblanco, Marie Curie 2, 28049 Madrid, Spain. Fax: +34-91-5854760. E-mail: fplou@icp.csic.es. URL:http://www.icp.csic.es/abgroup

Abstract

The *in vitro* fermentation properties of several purified galacto-oligosaccharides (GOS), specifically the trisaccharides 4'-galactosyl-lactose and 6'-galactosyl-lactose, and a mixture of the disaccharides 6-galactobiose and allolactose, was carried out. The bifidogenic effect of GOS at 1% (w/v) was studied in a pH-controlled batch culture fermentation system inoculated with healthy adult human faeces. Results were compared with those obtained with a commercial GOS mixture (Bimuno-GOS). Changes in bacterial populations measured through fluorescence *in situ* hybridization (FISH) and short-chain fatty acid (SCFA) production were determined. *Bifidobacteria* increased after 10 h fermentation for all the GOS substrates, but the changes were only statistically significant ($P < 0.05$) for the mixture of disaccharides and Bimuno-GOS. Acetic acid, whose formation is consistent with *bifidobacteria* metabolism, was the major SCFA synthesized. The acetate concentration at 10 h was similar with all the substrates (45-50 mM) and significantly higher than the observed for formic, propionic and butyric acids. All the purified GOS could be considered bifidogenic under the assayed conditions, displaying a selectivity index (SI) in the range 2.1-3.0, which was slightly lower than the determined for the commercial mixture Bimuno-GOS.

Keywords: Batch culture system, Galacto-oligosaccharides, Prebiotics, Transglycosylation, β-Galactosidase, FISH, Short-chain fatty acids.

Introduction

Human milk contains a complex mixture of proteins, lipids, enzymes, antibodies, nutrients and a variety of nondigestible oligosaccharides that provide an immunological protection to infants (Zivkovic et al. 2011). It is well reported that breast-fed infants harbor much higher level of *bifidobacteria* in their faecal microbiota than formula-fed infants due to their ability to utilize human milk oligosaccharides (HMOs, Charalampopoulos and Rastall 2009; Shen et al. 2011). HMOs stimulate the growth of *bifidobacteria* and *lactobacilli*, thus decreasing the faecal pH and reducing the presence of pathogens (Boehm et al. 2005). In this context, the intake of prebiotics such as HMOs influences the composition of the gut microbiota by increasing the proportion of these bacteria to other species. This change in bacterial populations causes a positive effect on human health as a consequence of immune stimulation, inhibition of pathogens, improved absorption of minerals, decrease of serum lipids concentration and reduction of cancer risk, among other effects (Gibson 1995; Gibson and Rastall 2006; Roberfroid 2007).

Among the HMOs present in human milk, galacto-oligosaccharides (GOS) constitute a major group (Shadid et al. 2007). The total GOS in human milk is approx. 8-12 g/l (Angus et al. 2007; Macfarlane et al. 2008) with 3'-, 4'- and 6'-galactosyl-lactose as the main components (Boehm et al. 2005). In the laboratory, GOS can be synthesized with β-galactosidases (EC 3.2.1.23) by transgalactosylation reactions in which lactose (or the glucose and galactose released by hydrolysis) serve as galactosyl acceptors yielding a series of di-, tri- and tetrasaccharides and eventually of higher polymerization degree (Park and Oh 2010; Torres et al. 2010). The GOS yield is determined by the balance between hydrolysis and transgalactosylation reactions (Hsu et al. 2007; Matella et al. 2006). Depending on the biological source of β-galactosidase, the yield and composition of GOS vary notably (Iqbal et al. 2010; Maischberger et al. 2010; Splechtna et al. 2006; Urrutia et al. 2013). The complete

identification of the GOS synthesized by a particular enzyme is a difficult task; in fact, considering the formation of β(1→2), β(1→3), β(1→4) and β(1→6) bonds, the number of possible linear GOS accounts for 7 disaccharides, 32 trisaccharides, 128 tetrasaccharides and so on. It is worth emphasizing that most of the previous *in vitro* studies to evaluate the fermentation selectivity of oligosaccharides have been carried out with complex mixtures (Sanz et al. 2005a; Shen et al. 2011) and only in a few cases with purified products (Cardelle-Cobas et al. 2011 and 2012). Therefore similar studies with purified individual GOS would be of great value to understand the effect of GOS composition and structure on their properties.

Fermentation selectivity can be estimated using *in vitro* fermentation systems inoculated with human faecal slurries (Sarbini et al. 2011; Shen et al. 2011). The growth of bacteria can be measured by fluorescence *in situ* hybridization (FISH) employing synthetic fluorescent oligonucleotide probes that targets specific regions of the 16S rRNA (Langendijk et al. 1995). One of the main functions of the microbiota is to salvage energy from nondigested carbohydrates in the upper gut. In particular, *Bifidobacterium* and *Lactobacillus* species are recognized as health-promoting and thus widely used as probiotics (Gomez et al. 2010; Mandalari et al. 2007; Vulevic et al. 2004). Concomitant to the increase of *bifidobacteria* and *lactobacilli*, short-chain fatty acids (SCFA) are produced by oligosaccharide fermentation (Charalampopoulos and Rastall 2009) and further absorbed, representing 40-50% of the available energy of the carbohydrate. The principal SCFA are acetate, propionate and butyrate, which are metabolized by the colonic epithelium (butyrate), liver (propionate) and muscle (acetate) (Cummings and Macfarlane 1997).

The aim of the present study was the initial *in vitro* evaluation of the potential prebiotic properties of several purified galacto-oligosaccharides with different linkages, and the results were compared with a commercial GOS mixture.

Materials and methods

Materials

The commercial β-galactosidases from *Bacillus circulans* (Biolactase) and *Kluyveromyces lactis* (Lactozym 3000 L HP G) were supplied by Biocon (Spain) and Novozymes A/S (Denmark), respectively. All nucleotide probes used for fluorescence *in situ* hybridization (FISH) were commercially synthesized and labelled with the fluorescent dye Cy3 (Sigma-Aldrich) at the 5´- position. All media and chemicals were purchased from Sigma-Aldrich, Oxoid and Fisher Scientific. Sterilization of media and instruments was done by autoclaving at 121°C for 15 min. The commercial mixture Bimuno-GOS was supplied by Clasado Ltd., with 52% GOS content.

Purification of GOS

The 4'-galactosyl-lactose was purified from the reaction mixture obtained with the β-galactosidase from *B. circulans* as described by Rodriguez-Colinas et al. (2012). From the reaction of lactose with β-galactosidase from *K. lactis,* 6'-galactosyl-lactose and a mixture of allolactose [Gal-β(1→6)-Glc] and 6-galactobiose [Gal-β(1→6)-Gal] were obtained according to the protocol described by Rodriguez-Colinas et al. (2011). For the purification of these products, the reactions were stopped when yield reached the maximum value for each desired oligosaccharide. Products were isolated by semipreparative hydrophilic interaction chromatography (HPLC-HILIC) using a quaternary pump (Delta 600, Waters) coupled to a LiChrospher-NH2 column (5 μm, 10 x 250 mm, Merck). The column temperature was kept constant at 30°C. Acetonitrile/water 90/10 (v/v), degassed with helium, was used as mobile phase at 6.25 ml/min for 8 min. Then, a gradient to acetonitrile/water 80/20 (v/v) was performed in 3 min, and this eluent was maintained during 6 min. Finally, a gradient to acetonitrile/water 75/25 (v/v) was performed in 3 min and maintained for 15 min. Peaks were

detected using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60°C. A three-way flow splitter (model Accurate, Dionex) and a fraction collector (Waters) were employed. The fractions containing the main peaks were pooled, and the solvent was eliminated by rotary evaporation using an ACTEvap evaporation module. Bimuno-GOS was treated with activated charcoal to reduce the content of monosaccharides following the method of Morales et al. (2006) with some modifications (Hernandez et al. 2009). Briefly, 500 mg of Bimuno-GOS and 3 g of activated charcoal (Darco G60, 100 mesh, Sigma-Aldrich) were mixed with 100 ml of ethanol (15% v/v) and stirred for 30 min. The mixture was filtered through Whatman No. 1 filters under vacuum and then the activated charcoal was washed with the ethanolic solution. Desorption of oligosaccharides from the activated charcoal was carried out with 100 ml of ethanol (50% v/v), stirred during 30 min and filtered as previously described. The filtrates were evaporated under vacuum at 40-45°C and filtered through 0.20 µm filters. Water was removed by freeze-drying.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The analysis of the purity of the isolated GOS was performed by HPAEC-PAD on a ICS3000 Dionex system (Dionex Corp., Sunnyvale, CA) consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 x 250 mm Carbo-Pack PA-1 column (Dionex) connected to a CarboPac PA-1 guard column was used at 30°C. For eluent preparation, MilliQ water and 50% (w/v) NaOH (Sigma-Aldrich) were used. The initial mobile phase was 15 mM NaOH at 1.0 ml/min for 12 min. A mobile phase gradient from 15 mM to 200 mM NaOH was established at 1.0 ml/min

for 15 min and the latter mobile phase was maintained for 25 min. The peaks were analyzed using Chromeleon software version 6.80. Identification of the different carbohydrates was done based on commercial GOS standards and other purified and characterized GOS (Rodriguez-Colinas et al. 2011 and 2012; Urrutia et al. 2013).

Faecal inocula

Experiments were carried out using fresh faecal samples from four healthy human donors (30 to 36 years old) who were free of any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics 6 months before faecal sample donation. Samples were diluted 1:10 (w/v) in sterile phosphate-buffered saline (PBS; pH 7.3) and homogenized in a stomacher (Stomacher 400, Seward) for 2 min at normal speed.

Batch culture fermentations

Sterile stirred batch culture fermentation vessels (5 ml working volume) were prepared and aseptically filled with 4.5 ml of sterile basal nutrient medium. This medium comprised of peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), KH_2PO_4 (0.04 g/l), $MgSO_4$ x 7 H_2O (0.01 g/l), $CaCl_2$ x 6 H_2O (0.01 g/l), $NaHCO_3$ (2 g/l), Tween 80 (2 ml/l), haemin (0.05 g/l), vitamin K (10 µl/l), L-cysteine hydrochloride (0.5 g/l) and bile salts (0.5 g/l). The basal medium was adjusted to pH 7.0 and then 4 ml of 0.025% (w/v) resazurin solution was added per liter before autoclaving. Once in the fermentation vessels, the sterile medium was sparged overnight with O_2-free N_2 (15 ml/min) to maintain anaerobic conditions. The following day, each vessel was inoculated with 0.5 ml of fresh faecal slurry prepared as described before. The GOS assayed were added at a concentration of 1% (w/v) to each vessel just prior to the addition of the faecal slurry. The temperature of the fermentation vessels was held at 37°C using a circulating water bath. The pH was maintained in the range of 6.6-6.9 via pH

controllers (Fermac 260, Electrolab) and adjusted by the addition of 0.5 mmol/l NaOH and HCl to the vessels when required. Anaerobic conditions were maintained throughout the fermentation by sparging the vessels with O_2 -free N_2 (15 ml/min). Batch culture fermentations were ran for 24 h and samples were taken at 0, 5, 10 and 24 h for analysis of bacterial populations by FISH and for analysis of SCFA by high-performance liquid chromatography (HPLC). Four fermentation experiments were performed, one per faecal donor.

Bacterial populations by FISH

Synthetic oligonucleotide probes targeting specific regions of the 16S rRNA molecule and labelled with a fluorescent dye (*Bif164, Lab158, Bac303, Ato291, Chis150, Erec482, Fpra655,* and *Eub 338*) were utilized for the enumeration of different bacterial groups (Table 1). FISH was performed as described by Martin-Pelaez et al. (2008).

Short-chain fatty acid (SCFA) analysis

Samples taken from the fermenters were centrifuged (13,000 g for 10 min) and supernatants were filtered through 0.22 µm syringe filters (Millex 13 mm filter units, Millipore). Samples (20 µl) were injected into an HPLC system equipped with refractive index and UV (210 nm) detectors put in series. The ion-exclusion column (Rezex ROA-organic acid, 300 x 7.8 mm, Phenomenex) was maintained at 82°C at a flow rate 0.5 ml/min. The eluent used was 2.5 mM sulphuric acid in HPLC-grade water. The sample run time was 40 min. SCFA quantification was carried out using calibration curve standards for formate, acetate, propionate and butyrate at concentrations of 12.5, 25, 50, 75 and 100 mM.

Statistical analysis

Differences between bacterial accounts and SCFA profiles at 0, 5, 10 and 24 h fermentation for each substrate were tested using SPSS program. Univariate analysis of variance (ANOVA) and post-hoc Tukey and Duncan tests were used to determine the significant differences of substrates. Differences were deemed significant at $P < 0.05$.

Results

Production and purification of GOS

Several GOS were obtained by transgalactosylation of lactose using two β-galactosidases (from *B. circulans* and *K. lactis*) with different specificity. In the case of *K. lactis* β-galactosidase, the enzyme exhibits a clear tendency to synthesize β-(1→6) bonds: the main products are the disaccharides 6-galactobiose [Gal-β(1→6)-Gal] and allolactose [Gal-β(1→6)-Glc], and the trisaccharide 6'-galactosyl-lactose [Gal-β(1→6)-Gal-β(1→4)-Glc] (Martínez-Villaluenga et al. 2008; Rodriguez-Colinas et al. 2011). In contrast, the *B. circulans* counterpart displays specificity for the formation of β(1→4) bonds; the major product synthesized was the trisaccharide 4'-galactosyl-lactose [Gal-β(1→4)-Gal-β(1→4)-Glc] (Rodriguez-Colinas et al. 2012). Fig. 1 shows the structures of the GOS assayed in this work. The products were purified in 250 mg-scale by semipreparative HPLC. It is noteworthy that the two disaccharides (6-galactobiose and allolactose) could not be efficiently separated by this technique and were tested as a "disaccharide mixture with β(1→6) bonds" in the fermentation assays.

The purity of the synthesized GOS was in the range 65-75% as determined by HPAEC-PAD (Fig. 2). All the samples showed the presence of variable amounts of galactose and glucose, as well as other minor oligosaccharides. Table 2 summarizes the different carbohydrates employed as substrates in the fermentation studies and their purity. Results were compared with a commercial prebiotic GOS (Bimuno-GOS) produced by *Bifidobacterium bifidum,* which was partially purified by activated charcoal to reduce the content of mono- and disaccharides. HPAEC-PAD analysis (Fig. 2) shows that Bimuno-GOS contains a complex mixture of oligosaccharides.

Bacterial enumeration

Batch pH-controlled fermentations were carried out under anaerobic conditions (Fig. 3) in the presence of the GOS synthesized to assess if the chemical structure of these products could influence bacterial selectivity. Results were compared with Bimuno-GOS used as a reference. Different hybridization probes (Table 1) were employed to count, by fluorescent microscopy, numerically dominant and physiologically relevant groups of bacteria. Bacterial counts (Table 3) were analyzed statistically for each substrate to determine which variations between the different fermentation times (0, 5, 10 and 24 h) could be considered significant.

As shown in Table 3, no substantial changes were observed in most bacterial populations during fermentation with the exception of *Faecalibacterium prausnitzii* (*Fpra655*) that decreased more than one $\log_{10}$ unit from the 0 h value with $P < 0.035$. *Bifidobacteria* (*Bif164*) increased significantly after 10 h fermentation for the mixture of disaccharides (6-galactobiose and allolactose) with $P < 0.01$ and for the control (Bimuno-GOS) with $P < 0.05$, whereas a statistically nonsignificant increment was observed for the trisaccharides 4'- and 6'-galactosyl-lactose. Interestingly, bifidobacterial counts at 24 h remained nearly constant with respect to 10 h fermentation. Therefore, all the GOS studied could be considered bifidogenic under the assayed conditions. In contrast, only a slight increase of *Lactobacillus* spp. population ($P > 0.05$) was observed after 10 h fermentation. *Bacteroides*, which comprises about 30% of the total colonic culturable microflora, maintained a stable population throughout the fermentation. In addition, purified GOS and Bimuno-GOS did not favour the growth of *Atopobium* spp. and *Eubacterium* spp. groups.

Short-chain fatty acids (SCFA) analyses

Table 4 summarizes the concentrations of formate, acetate, propionate and butyrate measured by HPLC in the faecal fermentation supernatants with the different substrates and

Bimuno-GOS as reference. The chromatographic analyses of SCFA were performed at the same time points (0, 5, 10 and 24 h) than the bacterial counts. After 24 h of fermentation, acetate was clearly the most prevalent SCFA with all the substrates tested, in agreement with the FISH results of *bifidobacteria* growth. The acetate concentration at 10 h was very similar with all the substrates (approx. 45-50 mM). Significant changes in propionate concentration throughout fermentation were observed with all the carbohydrates. The highest level (15 mM) corresponded to 4'-galactosyl-lactose at 5 h. However, the propionate concentration generally decreased after 5 h fermentation.

Low levels of butyrate were produced upon the fermentation of all the substrates. This can be related with the slight changes in the populations of *Clostridium coccoides-Eubacterium rectale* group, the major butyrate-producing bacterial groups found in human faeces (Barcenilla et al. 2000). The tested GOS increased formic acid concentration in the first five or ten hours of fermentation with a further decrease until 24 h, except for the mixture of disaccharides in which the formate concentration was maintained.

Discussion

The present study was carried out to evaluate the *in vitro* fermentation properties of several purified galacto-oligosaccharides (GOS), namely the trisaccharides 4'-galactosyl-lactose, 6'-galactosyl-lactose, and a mixture of the disaccharides 6-galactobiose and allolactose, which were selectively obtained by a proper selection of the β-galactosidase source.

Methods for the *in vitro* assessment of prebiotic properties range from the static batch cultures to the multiple-stage continuous cultures inoculated with either single/mixed bacterial strains or faecal homogenates (Tzortzis and Vulevic 2009). In our work, a batch culture fermentation system with a faecal homogenate was analyzed under anaerobic conditions and pH control. However, batch and continuous *in vitro* models do not provide a full picture of the complex ecosystem that exists within the large intestine.

In our study, the GOS assayed gave rise to an increase in *Bifidobacterium* populations, exhibiting certain degree of selectivity for this genus. Rycroft et al. (2001) reported that commercial GOS increased the level of *bifidobacteria* and lactic acid bacteria in human faecal batch culture fermentations, in some cases causing clostridia and bacteroides to decline. Sanz et al. (2005b) showed that bifidobacterial populations increased with several galactose-containing disaccharides (e.g. galactobioses, melibiose), whereas a slight increase without statistical significance was reported for *lactobacilli*. In a recent study, Cardelle-Cobas et al. (2012) showed that several oligosaccharides derived from lactulose were selectively fermented both by *Bifidobacterium* and lactic acid bacterial populations. The significant decrease of *Faecalibacterium prausnitzii* (*Fpra655*) that we observed with all substrates in the present work was also reported by Sarbini et al. (2011) when studying the fermentation properties of a series of linear and α(1→2)-branched dextrans.

To determine the global effect of the different GOS on the bacterial populations, the selectivity index (SI) was calculated at 10 h fermentation following the formula described by Ruiz-Matute et al. (2011):

$$SI = \frac{(Bif_{10}/Bif_0) + (Lac_{10}/Lac_0) + (Erec_{10}/Erec_0) - (Bac_{10}/Bac_0) - (Chis_{10}/Chis_0)}{(Total\ Count_{10}/Total\ Count_0)}$$

in which Bif_{10} and Bif_0 are the bifidobacterial counts at 10 h and 0 h, respectively, etc. SI values give an idea of the growth of beneficial faecal bacteria (*bifidobacteria*, *Lactobacillus/Enterococcus* group and *Eubacterium rectale* group) with respect to the less desirable ones (*Clostridium histolyticum* group and bacteroides). SI values are normalized by the total number of bacteria (determined with the *Eub* probes).

Fig. 4 shows that the SI values of the substrates assayed varied in the range 2.1-3.9. The highest value was obtained with Bimuno-GOS, which contains a mixture of GOS, thus suggesting the possibility of a synergistic effect between the different oligosaccharides or a strong effect of β(1→3) linkages present in this sample (Depeint et al. 2008). SI values were lower than those reported by Cardelle-Cobas et al. (2012) for lactulose-derived oligosaccharides or by Sanz et al. (2005b) for a series of galactobioses. However, SI values were similar to those described for honey oligosaccharides (Sanz et al. 2005a) or cellobiose-derived oligosaccharides (Ruiz-Matute et al. 2011). However, comparison of SI values must be performed with caution because significant differences can be encountered between the human donors (Cardelle-Cobas et al. 2012).

SCFA are produced as the end of oligosaccharide fermentation (Wang and Gibson 1993), and present positive implications in the prevention of colon cancer (Cummings 1981). The fermentation of all GOS substrates induced the production of acetate, which correlated with the increase of *Bifidobacterium* populations, as acetate formation is consistent with

bifidobacteria and *lactobacilli* metabolism (Macfarlane et al. 2008). The propionate production in all fermentations studied could be related with the slight –although statistically nonsignificant– increase of *Chys150* group, because several species within the *Clostridium histolyticum* group are able to produce propionate (Sarbini et al. 2012). The low concentration of butyrate could be related with the decrease of *Faecalibacterium prausnitzii* (Fpra655) populations determined by FISH.

In conclusion, the above results indicate that the GOS tested, which present different polymerization degree, composition and/or glycosidic linkages, display a similar *in vitro* fermentation selectivity towards the *Bifidobacterium* genus, which does not substantially differ from that observed with the GOS mixture Bimuno-GOS. The GOS assayed also promoted the decrease of *Faecalibacterium prausnitzii* group. The SCFA production correlated well with changes in bacterial populations; acetate was the most prevalent fatty acid formed. Interestingly, disaccharides containing β(1→6) linkages presented similar bifidogenic properties than the trisaccharides containing β(1→4) and β(1→6) bonds.

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Table 1. Probes used for FISH analysis of bacterial populations in samples from batch culture systems.

Probe	Specifity	Temperature (°C)		Sequence (5´ to 3´)	Reference
		Hybridization	Washing		
Bif164	*Bifidobacterium* spp.	50	50	CATCCGGCATTACCACCC	Langendijk et al. 1995
Lab158	*Lactobacillus-Enterococcus*	50	50	GGTATTAGCAYCTGTTTCCA	Harmsen et al. 1999
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	46	48	CCAATGTGGGGGACCTT	Manz et al. 1996
Ato291	*Atopobium* cluster	50	50	GGTCGGTCTCTAACCC	Harmsen et al. 2000
Chis150	Most of *Clostridium histolyticum* group (*Clostridium* cluster I and II)	50	50	TTATGCGGTATTAATCTYCCTTT	Franks et al. 1998
Erec482	Most of *Clostridium coccoides-Eubacterium rectale* group	50	50	GCTTCTTAGTCARGTACCG	Franks et al. 1998
Fpra655	*Faecalibacterium prausnitzii* and relatives	58	58	CGCCTACCTCTGCACTAC	Hold et al. 2003
Eub 338[a]	*Eubacterium* spp.	46	48	GCTGCCTCCCGTAGGAGT	Daims et al. 1999
Eub 338II[a]				GCAGCCACCCGTAGGTGT	
Eub 338III[a]				GCTGCCACCCGTAGGTGT	

[a] These probes are used together in equimolar concentration (all at 50 ng/µl)

Table 2. Substrates employed in this study to investigate the fermentation selectivity.

Degree of polymerization	**Substrate**	**Structure**	**Enzyme source**	**HPAEC-PAD purity (%)**
Trisaccharide	4'-Galactosyl-lactose	Gal-β(1→4)-Gal-β(1→4)-Glc	*B. circulans*	65
Trisaccharide	6'-Galactosyl-lactose	Gal-β(1→6)-Gal-β(1→4)-Glc	*K. lactis*	71
Disaccharides	Allolactose 6-Galactobiose	Gal-β(1→6)-Glc Gal-β(1→6)-Gal	*K. lactis*	76
Mixture	Bimuno-GOS	Contains mainly β(1→3) linkages [a]	*B. bifidum*	-

[a] Depeint et al. 2008

Table 3. Mean bacterial populations in pH-controlled batch cultures at 0, 5, 10 and 24 h. The starting concentration of the test substrates was 1% (w/v) of 5 ml batch culture fluid. Experiments were carried out using fresh faecal samples from four healthy human donors.

Probe/stain	Time point (h)	Bacterial population (log $_{10}$ cells/ml batch culture fluid)[a]			
		Allolactose + 6-Galactobiose	4'-Galactosyl-lactose	6'-Galactosyl-lactose	Bimuno-GOS
Bif 164	0	8.97 (0.33)	8.97 (0.33)	8.97 (0.33)	8.97 (0.33)
	5	8.62 (0.14)	8.67 (0.45)	8.57 (0.16)	8.57 (0.17)
	10	9.24 (0.10) **‡**	9.19 (0.32)	8.97 (0.46)	9.17 (0.21) ‡
	24	9.22 (0.20) **‡**	9.10 (0.17)	9.04 (0.27)	9.24 (0.11) **‡**
Lab158	0	7.81 (0.01)	7.81 (0.01)	7.81 (0.01)	7.81 (0.01)
	5	8.26 (0.37)	8.10 (0.45)	8.00 (0.64)	8.06 (0.51)
	10	8.00 (0.66)	8.00 (0.45)	8.05 (0.59)	8.14 (0.39)
	24	7.88 (0.66)	8.04 (0.49)	8.10 (0.53)	7.99 (0.59)
Bac303	0	9.09 (0.19)	9.09 (0.19)	9.08 (0.19)	9.09 (0.19)
	5	8.60 (0.25) **†**	8.73 (0.22) †	8.68 (0.19)	8.66 (0.27)
	10	8.88 (0.11)	8.97 (0.10)	9.06 (0.08)	9.00 (0.33)
	24	9.03 (0.08) ‡	9.25 (0.12) **‡**	9.20 (0.31) ‡	9.03 (0.25)
Ato291	0	8.78 (0.12)	8.78 (0.12)	8.78 (0.12)	8.78 (0.12)
	5	8.42 (0.29)	8.39 (0.10) †	8.33 (0.60)	8.34 (0.30)
	10	8.53 (0.16)	8.76 (0.34) ‡	8.46 (0.34)	8.62 (0.25)
	24	8.61 (0.19)	8.80 (0.27) ‡	8.65 (0.27)	8.69 (0.21)
Chis150	0	8.58 (0.15)	8.58 (0.15)	8.58 (0.15)	8.58 (0.15)
	5	8.79 (0.60)	8.91 (0.43)	8.92 (0.35)	8.80 (0.42)
	10	8.88 (0.38)	8.84 (0.47)	8.40 (0.51)	8.68 (0.61)
	24	9.14 (0.08)	8.79 (0.56)	8.97 (0.35)	8.66 (0.59)
Erec482	0	9.39 (0.05)	9.39 (0.05)	9.39 (0.05)	9.39 (0.05)
	5	9.09 (0.08) **†**	9.04 (0.21)	8.67 (0.81)	9.08 (0.15)
	10	9.25 (0.13)	9.07 (0.27)	9.15 (0.21)	9.23 (0.12)
	24	9.22 (0.13)	9.08 (0.70)	9.05 (0.14)	9.09 (0.30)
Fpra655	0	8.87 (0.20)	8.87 (0.20)	8.87 (0.20)	8.87 (0.20)
	5	7.84 (0.08) **†**	7.91 (0.12)	7.78 (0.22) **†**	7.90 (0.13) **†**
	10	7.17 (0.06) **† , ‡**	7.74 (0.56) †	7.31 (0.16) **†**	7.32 (0.50) **†**
	24	7.06 (0.40) **† , ‡**	7.28 (0.80) **†**	7.04 (0.62) **†**	7.30 (0.44) **†**
Eub338	0	9.68 (0.17)	9.68 (0.17)	9.68 (0.17)	9.68 (0.17)
	5	9.34 (0.19) †	9.38 (0.13)	9.29 (0.32)	9.45 (0.06)
	10	9.49 (0.12)	9.40 (0.23)	9.44 (0.08)	9.46 (0.20)
	24	9.54 (0.13)	9.67 (0.12)	9.51 (0.21)	9.69 (0.25)

[a] Standard deviations are shown in parenthesis

† Significant difference from the 0 h value for the same substrate with $P < 0.05$; ‡ Significant difference from the 5 h value for the same substrate with $P < 0.05$; In bold type: significant differences with $P < 0.01$.

Table 4. Short-chain fatty acid (SCFA) concentrations in pH-controlled batch cultures at 0, 5, 10 and 24 h. Mean values were calculated from experiments carried out using fresh faecal samples from four healthy human donors.

SCFA	Time point (h)	Mean SCFA concentrations (mM)[a]			
		Allolactose + 6-Galactobiose	**4'-Galactosyl-lactose**	**6'-Galactosyl-lactose**	**Bimuno-GOS**
Formate	0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	5	9.41 (2.61)**†**	5.10 (1.07) **†**	8.74 (3.42) †	6.87 (1.20) **†**
	10	10.2 (3.6) **†**	14.3 (0.7) **†, ‡**	11.4 (4.1) **†**	4.91 (1.49) **†**
	24	9.31 (2.62) **†**	0.00 (0.00) **‡, §**	1.43 (0.95) §	3.57 (0.04) **†**, ‡
Acetate	0	0.40 (0.11)	0.40 (0.11)	0.40 (0.11)	0.40 (0.11)
	5	22.6 (3.4)	19.0 (3.0) **†**	22.0 (4.2) **†**	21.9 (4.0)
	10	49.8 (5.6) **†**, ‡	45.2 (0.2) **†, ‡**	49.7 (6.9) **†, ‡**	49.6 (12.1) **†**, ‡
	24	78.7 (17.9) **†, ‡, §**	60.0 (6.2) **†, ‡**, §	18.2 (0.4) †, **§**	99.9 (4.8) **†, ‡, §**
Propionate	0	0.18 (0.05)	0.18 (0.05)	0.18 (0.05)	0.18 (0.05)
	5	3.13 (0.06) †	15.0 (1.6) **†**	5.06 (0.23) **†**	7.85 (1.33) **†**
	10	3.08 (0.61) †	3.71 (0.82) **‡**	3.03 (0.58) **†**, ‡	1.42 (0.19) **‡**
	24	4.84 (1.33) **†**	5.89 (3.18) **‡**	3.93 (0.76) **†**	9.52 (0.14) **†, §**
Butyrate	0	0.32 (0.02)	0.32 (0.02)	0.32 (0.02)	0.32 (0.02)
	5	3.06 (0.15) **†**	2.76 (0.47) †	2.04 (0.92)	3.45 (0.66)
	10	1.94 (0.47) †	2.05 (0.40)	1.68 (0.91)	2.75 (0.83)
	24	3.78 (0.42) **†, §**	4.62 (1.13) **†**, §	3.34 (1.65)	8.51 (2.12) **†**

[a] Standard deviations are shown in parenthesis

† Significant difference from the 0 h value for the same substrate with $P < 0.05$

‡ Significant difference from the 5 h value for the same substrate with $P < 0.05$

§ Significant difference from the 10 h value for the same substrate with $P < 0.05$

In bold type: significant differences with $P < 0.01$.

Figure Legends

Fig. 1. Structure of the GOS assayed in this study: (A) 4'-Galactosyl-lactose; (B) 6'-Galactosyl-lactose; (C.1) Allolactose; (C.2) 6-Galactobiose.

Fig. 2. HPAEC-PAD analysis of the GOS assayed: (A) 4'-Galactosyl-lactose; (B) 6'-Galactosyl-lactose; (C) Mixture of allolactose and 6-galactobiose; (D) Bimuno-GOS after treatment. Peaks: (1) Galactose; (2) Glucose; (3) 6-Galactobiose ; (4) Allolactose; (5) Lactose; (6) 6'-Galactosyl-lactose; (7) 4'-Galactosyl-lactose; (8) Gal-β(1→4)-Gal-β(1→3)-Glc; (9) Gal-β(1→4)-Gal-β(1→4)-Gal-β(1→4)-Glc.

Fig. 3. Scheme of the fermentation vessels employed in the pH-controlled faecal batch cultures: (1) Gas outlet; (2) N_2 inlet; (3) pH electrode; (4) Base feeder; (5) Acid feeder; (6) H_2O inlet at 37°C; (7) H_2O outlet.

Fig. 4. Selectivity index (SI) scores of the different GOS determined from pH-controlled batch culture fermentations (10 h).

Fig. 1

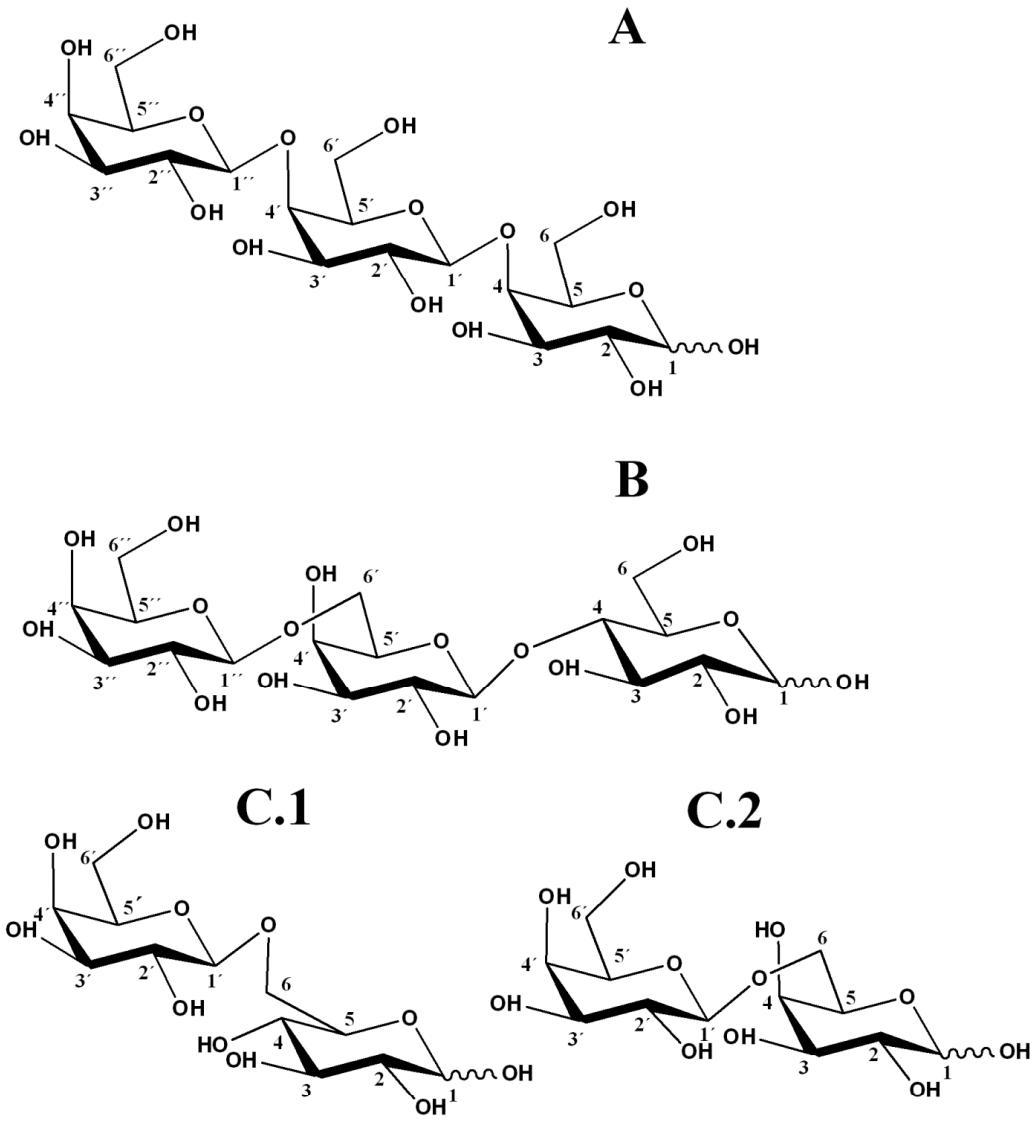

Fig. 2

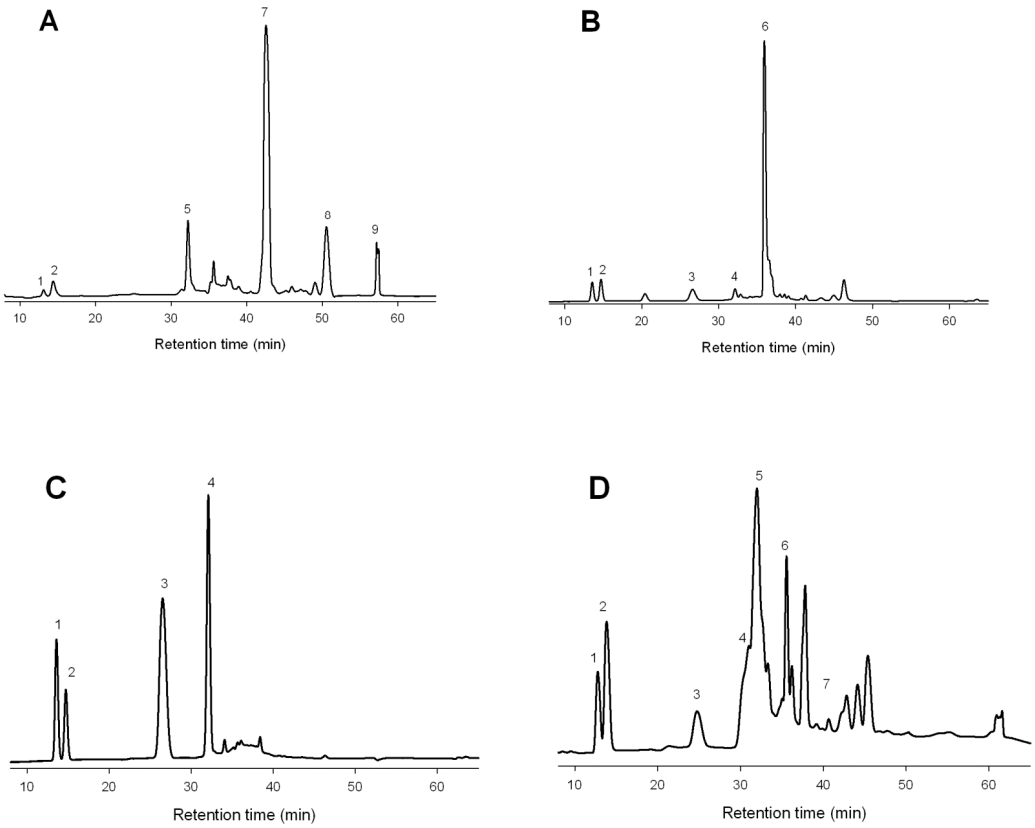

Fig. 3

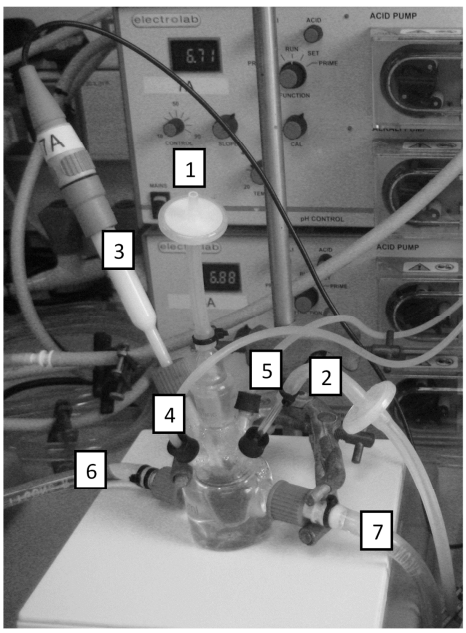

Fig. 4